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Production of a Biosurfactant from *Torulopsis bombicola*

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Two types of carbon sources—carbohydrate and vegetable oil—are necessary to obtain large yields of biosurfactant from *Torulopsis bombicola* ATCC 22214. Most of the surfactant is produced in the late exponential phase of growth. It is possible to grow the yeast on a single carbon source and then add the other type of substrate, after the exponential growth phase, and cause a burst of surfactant production. This product is a mixture of glycolipids. The maximum yield is 70 g liter^{-1}, or 35% of the weight of the substrate used. An economic comparison demonstrated that this biosurfactant could be produced significantly more cheaply than any of the previously reported microbial surfactants.

Large yields of glycolipids have been obtained from several species of *Torulopsis* yeasts grown for 3 to 7 days in shake flasks (3, 6, 10, 11, 14, 15). Several related glycolipids have been characterized in the crude extract. These all contain the dimeric sugar sophorose and a long-chain carboxylic acid with a hydroxyl function on the penultimate or terminal carbon. In most of the structures the acid is attached to the carbohydrate by the hydroxyl group, leaving a free carboxyl function. Recently, it has been demonstrated that these lipids have biosurfactant properties (8, 9).

A variety of biosurfactants have been characterized from bacteria (1–5, 12, 13). The sophorose lipids are one of the few examples of biosurfactants characterized from yeasts. From the published results of shake flask scale growth experiments, it appeared that the biosurfactant from *Torulopsis* species was produced in better yields than those from bacteria.

In this paper we consider the larger-scale production, characterization, and economic feasibility of surfactants from *Torulopsis bombicola* ATCC 22214.

MATERIALS AND METHODS

T. bombicola ATCC 22214 was maintained on yeast malt agar at 4°C and transferred at regular intervals. The standard medium used for the preliminary studies and the fermentation studies contained 0.1% KH_2PO_4, 0.5% $MgSO_4$, 0.01% $CaCl_2$, and 0.01% NaCl. Additions to this medium included yeast extract (Difco Laboratories), peptone (Difco), urea, NH_4Cl, NH_4NO_3, $NaNO_3$, glucose, or vegetable oil (or a combination of these). The oils were food grade, without preservatives, and included corn, soya bean, safflower, and sunflower oils.

The fermentations were carried out in a 7-liter New Brunswick Scientific Microferm fermentor. The air flow rate was 5 liters/min^{-1}, the temperature was 30°C, and two flat-blade turbines were rotated at 500 rpm.

Biomass was determined as dry weight. Fifty-milliliter samples containing vegetable oil were extracted with ethyl acetate and centrifuged twice, and the cell pellets were collected and dried at 90°C.

Surface tension, interfacial tensions, and critical micelle concentrations were all determined with a Fisher Autotensiomat (Fisher Scientific Co.) (1, 2, 4, 5).

Emulsification tests were done with test tubes containing water and hydrocarbon or vegetable oil as previously described (1).

Most of the glycolipid was removed as a lower oily phase. To ensure complete removal, the samples were extracted twice with equal volumes of ethyl acetate. The solvent was removed under reduced pressure. The crude product was washed three times with equal volumes of hexane, which was virtually immiscible with the oil, and weighed.

Several solvent systems were used to develop the thin-layer chromatographs. All of the reported data are for chloroform-methanol-water, 65:15:2 (vol/vol). The plates were Fisher Redi-Gel silica gel GF. Components were visualized by using a solution of α-napthol.

The method of analysis of bitumen release from the tar sands (4) has been reported previously (16). The floating bitumen was collected with 25 ml of toluene. The optical density of this sample was measured at 700 nm and compared with a calibration curve.

RESULTS

Effect of media on growth and glycolipid production. In preliminary shake flask experiments, the optimum medium for glycolipid production was determined. Yeast extract was found to be essential for growth. Table 1 shows typical results with media containing only glucose as a carbon source. All of the media supported growth, but only trace amounts of glycolipid were produced. Nitrate is not as good a nitrogen source for biomass production as the reduced nitrogen species.

Table 2 contains the averaged results from some of the shake flask experiments with mixed media containing ammonium nitrate. There was some variation of the biomass yield with the nature of the carbon source. However, the important change was the concentration of glycolipids. There was little glycolipid produced unless both glucose and vegetable oil were present. Again, yeast extract was found to be important for growth and glycolipid production. No further enhancement was observed for yeast extract additions greater than 0.5%, and this concentration was used for subsequent experiments. Peptone could be substituted for yeast extract, but gave only about half the yield of biomass and glycolipid. If urea was used instead of yeast extract, growth was poor and very little glycolipid was produced.

Table 3 shows that various vegetable oils could be used in the standard medium with similar results. In particular, the yield of glycolipid was essentially unaffected.

Fermentation studies. Figure 1 shows data from a typical 7-

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TABLE 1. Effect of nitrogen source on *T. bombicola*[a]

Nitrogen source[b]	Biomass (g/liter)	Surface tension[c] (mN m^{-1})	Interfacial tension[c] (mN m^{-1})
$NaNO_3$	4.82	33	1.8
NH_4Cl	9.61	31	1.5
NH_4NO_3	9.16	33	1.8
$(NH_2)_2CO$	10.14	34	1.8

[a] *T. bombicola* cultures were grown for 4 days on media containing 4% glucose and 0.1% yeast extract. Trace amounts of glycolipid were observed in each example.
[b] In each case, there was 0.05 mol of nitrogen in medium.
[c] Measurement of media after 4 days.

TABLE 2. Effect of medium composition on production of biosurfactant from *T. bombicola*

Glucose (%)	Safflower oil (%)	0.1% Yeast extract		0.5% Yeast extract	
		Biomass (g/liter)	Glycolipid (g/liter)	Biomass (g/liter)	Glycolipid (g/liter)
100	0	3.6	1.0	10.7	tr
100	50	6.6	3.0	13.5	10
100	100	6.1	5.0	16.8	30
50	100	7.5	10.0	12.4	18
0	100	4.1	1.0	12.7	tr

liter fermentation of *T. bombicola* in an optimum medium with a dilute inoculum. The biomass curve compared with the yield of glycolipid at various times shows that most of the biosurfactant is produced in the late exponential growth phase. The final yield of product was typically about 70 g $liter^{-1}$. This corresponds to 35% conversion of the added substrate on a weight basis.

Fermentations with the standard medium containing only glucose or only vegetable oil yielded very little product. This is demonstrated by the first part of Fig. 2, up to 90 h.

Experiments were done starting initially with only glucose or vegetable oil and then adding the second substrate after exponential growth was over. An example of this is the

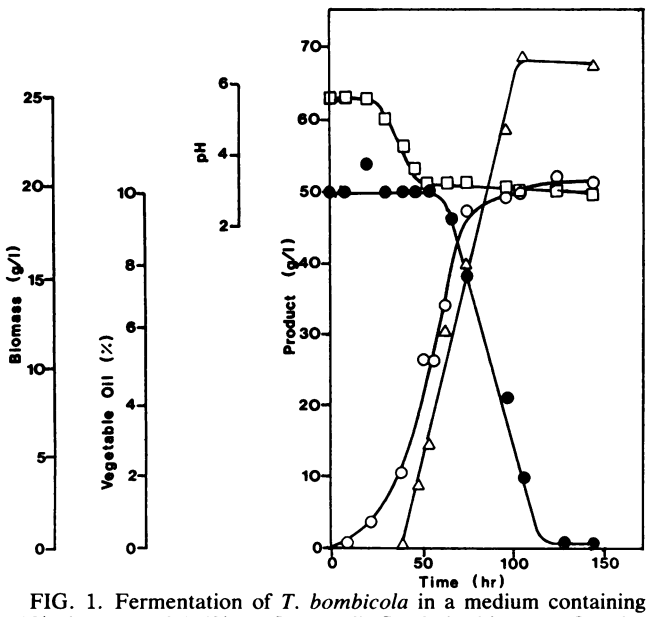

FIG. 1. Fermentation of *T. bombicola* in a medium containing 10% glucose and 9.5% sunflower oil. Symbols: biomass (○), glycolipid product (△), pH (□), and vegetable oil remaining (●).

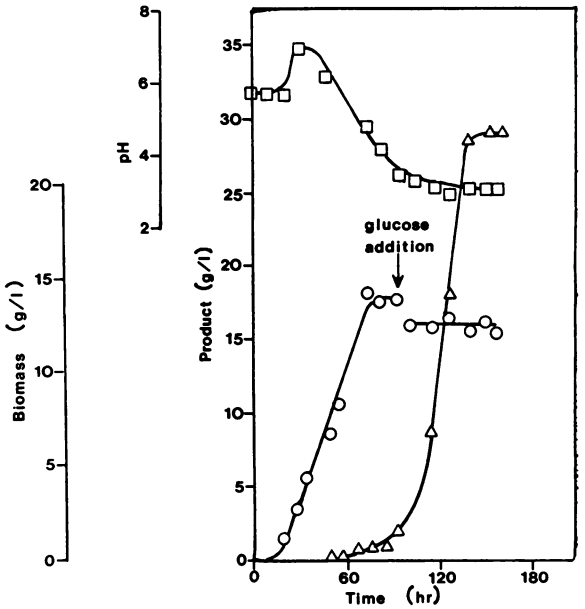

FIG. 2. Fermentation of *T. bombicola* in a medium containing 9.5% sunflower oil with addition of glucose at 90 h. Symbols: biomass (○), glycolipid product (△), and pH (□).

fermentation represented by Fig. 2. At 90 h a concentrated sterile glucose solution was added, resulting in 10% glucose in the fermentor. Within a few hours, there was a dramatic surge in the production of glycolipid. Analogous results were obtained when the initial fermentation medium contained only glucose and a vegetable oil was added after the active growth phase was over.

Characterization of the biosurfactant. After several washings with hexane, the product oil was found by thin-layer chromatography to contain six α-napthol-positive components. The R_f values for these six glycolipids, in order of decreasing relative concentration, were as follows: 0.62, 0.52, 0.60, 0.50, 0.39, and 0.19. No other spots were observed after treating the plates with ninhydrin, phospray (Supelco, Inc.), or after acid charing.

The same six components in the same relative order of concentration were observed for all the media used. This included different vegetable oils, mixed carbon sources, glucose-only media, different nitrogen sources, etc. Only product yield was dependent on the substrate used.

Surfactant properties of the biosurfactant were also unaffected by the media used. Figure 3 demonstrates the identical curves for plots of surface tension versus the log of the concentration of biosurfactant added to distilled water for media containing glucose and sunflower oil or glucose and safflower oil.

At pH 4 the critical micelle concentration of the biosurfactant was 82 mg $liter^{-1}$, and the minimum surface tension was

TABLE 3. Growth of *T. bombicola* on various vegetable oils

Vegetable oil[a]	Biomass (g/liter)	Glycolipid (g/liter)
None	10.7	tr
Safflower	12.4	18
Corn	15.1	20
Soya bean	14.3	18
Sunflower	15.6	17

[a] Oil and glucose concentrations were 10% by weight.

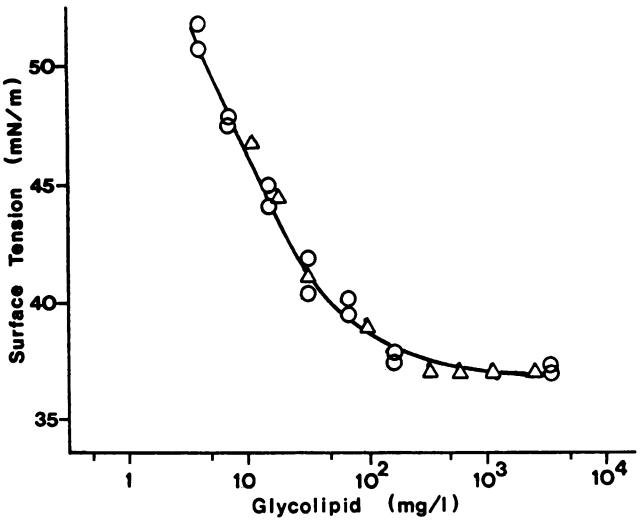

FIG. 3. Surface tension versus weight per volume of glycolipid added to distilled water. Symbols: lipid from sunflower oil (○) and from safflower oil (△).

37 mN m^{-1}. The minimum interfacial tensions against hexadecane or any of the substrate vegetable oils were all between 1 and 2 mN m^{-1}.

The surface tension and interfacial tension were insensitive to concentrations of either NaCl or $CaCl_2$ up to 100 g $liter^{-1}$.

The solubility of the glycolipids in water was pH dependent. Above pH 6 the mixture was 2 orders of magnitude more soluble than at pH 4.

The lipid mixture was not able to stabilize emulsions of water and hydrocarbon or water and vegetable oil.

The glycolipid was used to release bitumen from tar sand. Table 4 contains data for the amount of bitumen released to the surface, divided by the corresponding control value.

TABLE 4. Bitumen released from tar sand by glycolipids

Concn of biosurfactant (mg/liter)	Relative bitumen released to surface
1,100	8.0
110	4.6
11	2.0
0	1.0

DISCUSSION

The yield of biosurfactant from *T. bombicola* is dependent on the media used. When a complex medium containing both a carbohydrate and a vegetable oil was used, very high yields, up to 70 g $liter^{-1}$, could be obtained. Using only one of the carbon sources resulted in very little product.

The thin-layer chromatographic studies indicated that the insoluble oil was a mixture of glycolipids, as reported previously (3, 6, 10, 11, 14, 15). Although the yield of this biosurfactant was dependent on the nature of the substrate, the types of glycolipids and their relative concentrations showed little or no variation. This does not imply that the distribution of fatty acids in these lipids was identical, as thin-layer chromatography does not indicate these distinctions. It is likely that the substrate does influence the distribution of fatty acids, as has been observed for *Torulopsis* species and other microorganisms (3, 10, 11, 15).

This work has concentrated on the properties of the mixture of glycolipids. Some work has been done to characterize purified biosurfactants to be used in enhanced oil recovery (12). It would not be economical to isolate individual components from the *T. bombicola* product or any other biosurfactant to be used for surfactant applications. The purification costs would be prohibitive. The mixture from *T. bombicola* is easy to isolate and could be produced cheaply.

The glycolipids caused substantial lowering of surface tensions or interfacial tensions, but were not able to stabilize either water-hydrocarbon or water-vegetable oil emulsions. However, previous workers have shown that the sophorolipids improve the rate of growth of *Torulopsis* species on water-insoluble substrates (7, 9). The usual conclusion that the compounds are acting as emulsifiers cannot be correct. It is possible that the glycolipids do cause some dispersion. It was observed, qualitatively, that the lipid mixture and vegetable oil mutually improve dispersion into water of these immiscible phases in a stirred vessel.

The glycolipids can affect liquid-liquid interfaces as shown by the low interfacial tensions. The mixture can also act on solid-liquid interfaces as demonstrated by the action on tar sand. The lipids from *T. bombicola* caused significant release of bitumen from the sand.

Table 5 is a summary of the data for optimum yields of the glycolipids from *T. bombicola* compared with that for biosurfactant production from the literature. *T. bombicola* is an exceptionally good producer of biosurfactants. Also included in Table 5 is the estimated cost of producing various biosurfactants using the cheapest reasonable substrates. Again *T. bombicola* is the most promising organism. The estimated cost of production of the glycolipids ($2.75 per kg) is comparable to the cost of production of synthetic surfactants. These estimated costs will vary; but the relative differences are significant and demonstrate the importance of high yield and low substrate costs for commercial biosurfactant production to be feasible.

It was possible to produce biomass on either carbohydrate or vegetable oil substrate and then add the other substrate and produce the glycolipid without appreciably increasing the biomass. It will be possible to produce biomass with the cheapest available substrate and then with appropriate sub-

TABLE 5. Comparison of biosurfactants

Organism	Reference(s)	Substrate	Final product concn (g/liter)	% Yield (per g of substrate)	Cost[a] per kg
Corynebacterium lepus	4, 5	Kerosene	0.35	1.1	152.40
Rhodococcus erythropolis	12, 13	Alkanes	2.1	10.5	17.00
Bacillus subtilis	2	Glucose	0.8	2.0	17.20
T. bombicola		Glucose, soya bean oil	67	34.7	2.75
T. bombicola		Glucose	1.0	1.0	

[a] 1982 Canadian dollars, based on raw material costs as 35% of total cost and assuming kerosene, molasses, or soya bean oil as substrates. Compare with the cost of span 60 (synthetic surfactant, Atlas Chemical Industries) at $3.25 per kg.

strate additions produce the glycolipid. As the glycolipid is insoluble at the pH of the fermentations, it will be possible to continuously add substrate and remove glycolipid.

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